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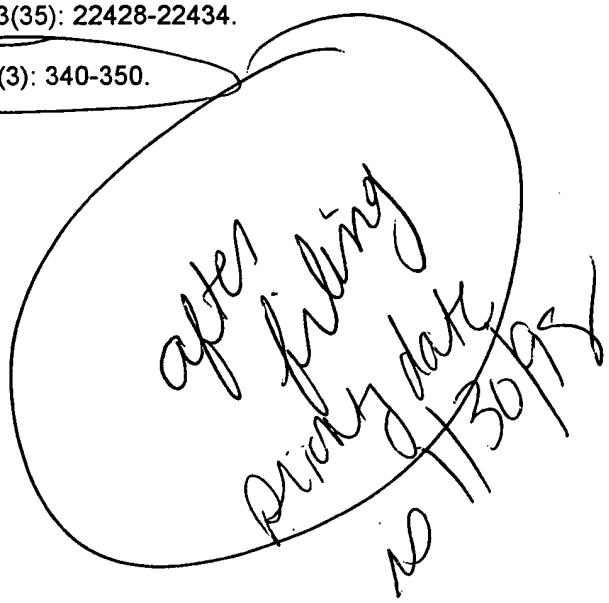

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New Eukaryotic Semaphorins with Close Homology to Semaphorins of DNA Viruses

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Semaphorins were initially described as a family of repulsive guidance molecules in embryonal development. Their basic structure consists of an N-terminal signal sequence, the defining semaphorin domain of approximately 500 amino acids, an Ig-like domain, and a variable carboxy-terminus. We recently described a viral semaphorin homologue encoded by the alcelaphine herpesvirus type 1. Less conserved, truncated homologues were also identified in poxviruses. Here we describe new human and murine semaphorin homologues. The respective genes were cloned and sequenced, and they were termed H-Sema-L and M-Sema-L (HGMW-approved symbols SEMAL and Semal, respectively). A multiply spliced mRNA of 3.2 kb is expressed in human placenta, spleen, thymus, and gonadal tissue. H-Sema-L maps to chromosome 15q22.3–q23 and M-Sema-L to the homologous locus 9A3.3-B in the mouse genome. The expression patterns and the presence of related genes in large DNA viruses suggest that this new semaphorin has a relevant function in the immune system. © 1998 Academic Press

INTRODUCTION

Growth cone guidance in the developing nervous system is tightly controlled by repulsive and attractive signals (Keynes and Cook, 1995; Culotti and Kolodkin, 1996; Mueller *et al.*, 1996). Repulsive signals are provided by the semaphorin family of proteins first described in chicken (Luo *et al.*, 1993), insects, and human (Kolodkin *et al.*, 1992, 1993). The semaphorins are characterized by the semaphorin (Sema) domain of approximately 500 amino acids, which contains about 14 conserved cysteine residues. An amino-terminal signal sequence indicates posttranslational processing of the proteins, and an Ig-like domain and a variable carboxy-terminus usually follow the Sema domain. Depending on the carboxy-terminus, there are secreted and membrane-associated forms, as well as membrane-associated forms with putative intracellular signaling domains. We recently described a viral semaphorin homologue in the alcelaphine herpesvirus type 1 (AHV-1) (Ensser and Fleckenstein, 1995; Ensser *et al.*, 1997). A less conserved, truncated homologue was also noted in poxviruses (Kolodkin *et al.*, 1993). The human CD100 molecule, a 150-kDa glycoprotein dimer expressed on resting and activated T-cells, is a semaphorin. It may play a role in modifying the interaction with B-cells by augmenting interactions between CD40 and its ligand CD40L (Hall *et al.*, 1996). Furthermore, the CD45 phosphatase plays a role in the modulation of the CD100 molecule (Herold *et al.*, 1996). The murine transmembrane semaphorin G (M-Sema-G) is also highly expressed in lymphoid tissues (Furuyama *et al.*, 1996). Two other human semaphorins have been identified at the genomic locus 3p21.3, a region associated with deletions in small cell lung cancer (Sekido *et al.*, 1996; Xiang *et al.*, 1996; Roche *et al.*, 1996), with expression in a variety of nonneural tissues. Currently the gene family has more than 30 homologues from insects, nematodes, vertebrates, and viruses (Table 1). The presence of semaphorin homologues in two different virus families, taken together with the discovery of M-Sema-G and the T-cell semaphorin CD100, hints at specific functions in the immune system.

Receptors for human and rat Sema-III have recently been described from the neuropilin family of proteins (Kolodkin *et al.*, 1997; He and Tessier-Lavigne, 1997). Neuropilin-1 is highly conserved among different species. The Sema domain and the carboxy-terminal basic domain of H-Sema-III bind to the extracellular domains of human neuropilin-1. The Ig-like domain is not necessary for binding neuropilin-1 (Kolodkin *et al.*, 1997). The phenotype of chimeric mice overexpressing murine neuropilin-1 is characterized by excess capil-

Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AF030697 (genomic (H-Sema-L), AF030698 (H-Sema-L cDNA), and AF030699 (M-Sema-L cDNA).

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TABLE 1

Name[a]	Synonym	Species	Comments	Reference or Accession No.
H-Sema-III	(H-Sema-D)	Human	S	Kolodkin *et al.* (1993)
CD-100		Human	TM, IC; CD45 asscociated, expressed in T-cells	Hall *et al.* (1996)
H-Sema-V	(H-Sema-A)	Human	S; Locus 3p21.3	Sekido *et al.* (1996); Roche *et al.* (1996)
H-Sema-IV	(H-Sema-3F)	Human	S; Locus 3p21.3	Xiang *et al.* (1996); Sekido *et al.* (1996)
H-Sema-E		Human	S; divergent 3′ end to M-Sema-E (frameshift corrected in alignment)	AB000220; Yamada (unpublished results)
H-Sema-K	KIAA0331	Human	S	Nagase *et al.* (1997)
H-Sema-L	SEMAL	Human	TM, no IC	This paper
M-Sema-A		Mouse	S	Püschel *et al.* (1995)
M-Sema-B		Mouse	TM, IC	Püschel *et al.* (1995)
M-Sema-C		Mouse	TM, IC	Püschel *et al.* (1995)
M-Sema-D	M-Sema-III	Mouse	S	Messersmith *et al.* (1995); Püschel *et al.* (1995)
M-Sema-E		Mouse	S; 5′ partial sequence	Püschel *et al.* (1995)
M-Sema-F1	M-Sema-F	Mouse	TM, IC	Inagaki *et al.* (1995)
M-Sema-G2	M-Sema-G	Mouse	TM, IC; expressed in lymphoid cells, murine CD100 homologue	Furuyama *et al.* (1996)
M-Sema-F2	M-Sema-F	Mouse	TM, IC; Thromospondin-motifs	Adams *et al.* (1996)
M-Sema-G1	M-Sema-G	Mouse	TM, IC; Thromospondin-motifs	Adams *et al.* (1996)
M-Sema-H		Mouse	S	Z80941; Christensen (unpublished results)
M-Sema-VIa		Mouse	TM, IC	Zhou *et al.* (1997)
M-Sema-L	Semal	Mouse	partial sequence	This paper
Collapsin-1		Chicken	S	Luo *et al.* (1993)
Collapsin-2		Chicken	S	Luo *et al.* (1995)
Collapsin-3		Chicken	S	Luo *et al.* (1995)
Collapsin-4		Chicken	partial sequence	Luo *et al.* (1995)
Collapsin-5		Chicken	S	Luo *et al.* (1995)
R-Sema-III		Rat	S	Giger *et al.* (1996)
T-Sema-I		*Tribolium confusum*	TM, IC	Kolodkin *et al.* (1993)
Ce-Sema-I		*C. elegans*	TM, IC	U15667; Roy (unpublished results)
G-Sema-I	Fasciclin-IV	Grashopper	TM, IC	Kolodkin *et al.* (1992)
D-Sema-I		*Drosophila*	TM, IC	Kolodkin *et al.* (1993)
D-Sema-II		*Drosophila*	S	Kolodkin *et al.* (1993)
AHV-Sema		AHV-1	S	Ensser and Fleckenstein (1995)
ORF-A39		Vaccinia	S	Kolodkin *et al.* (1993)
ORF-A39-homologue		Variola	S; frameshift errors in sequence	Kolodkin *et al.* (1993)

Note. The published semaphorins or those accessible from the databases that were used in the phylogenetic analysis (Fig. 4). The H-Sema-E sequence contained an apparent frameshift error at position 2185 in the sequence in the database. After comparison with the M-Sema-E sequence, a fifth adenine residue was added to a stretch of four, and this restored a reading frame highly conserved with M-Sema-E. There is a problem in the nomenclature of designation of M-Sema-F and -G, as the names have been used twice for different genes. We numbered them in order of publication M-Sema-F1, F2, G1, and G2, respectively.

[a] Designation used in phylogenetic tree (Fig. 4); TM, has putative membrane spanning sequence motif; IC, has putative intracellular cytoplasmatic sequence motif.

laries and blood vessels, dilation of blood vessels, malformed hearts, ectopic sprouting and defasciculation of nerve fibers, and extra digits (Kitsukawa *et al.*, 1995). This phenotype is similar to that of the knockout mouse lacking M-Sema-III (Behar *et al.*, 1996) and clearly supports a functional, inhibitory interaction of the M-Sema-III homologue with murine neuropilin 1. Another protein family found closely associated with semaphorin-mediated signaling is related to the dihydropyrimidinases (Hamajima *et al.*, 1996), such as the collapsin response mediator protein (CRMP) or unc-33-related proteins. These molecules are thought to be involved in intracellular signaling in response to binding of semaphorin to its receptor (Goshima *et al.*, 1995; Wang and Strittmatter, 1996).

In this paper we present new human and murine homologues closely related to the viral semaphorin genes. The unique structure and tissue-specific expression suggest distinct functions of the H-Sema-L[3] gene and its murine homologue.

MATERIALS AND METHODS

Polymerase chain reaction (PCR) applications. For the rapid amplification of cDNA ends (RACE), the Marathon Kit (Clontech, Heidelberg, Germany) was used with poly(A) RNA from placenta as starting material. PCR amplification of H-Sema-L cDNA was accom-

[3] The HGMW-approved symbols for the gene described in this paper are SEMAL for the human gene and Semal for the murine gene.

plished using 100 ng double-stranded cDNA generated from the placental poly(A) RNA. The genomic DNA fragments were amplified from 250 ng human genomic DNA, which was isolated from PHA-stimulated peripheral blood lymphocytes from a voluntary donor. Short fragments smaller than 1 kb were amplified with AmpliTaq (Perkin–Elmer, Weilderstadt, Germany). Larger fragments were amplified with the Expand Long Template PCR System (Boehringer Mannheim, Germany). PCR was performed on a MJ Research PTC-200 thermal cycler in thin-walled 0.2-ml tubes (Biozym, Hessisch-Oldendorf, Germany). For PCR with AmpliTaq, 35 to 40 cycles were performed at 96, 60, and 70°C for 15, 20, and 60 s, respectively; an initial denaturation step at 96°C for 1 min and a final extension step at 70°C for 1 min were included. For PCR and RACE with the Expand Long Template PCR System, 30 to 35 cycles were performed at 94, 62, and 68°C for 10, 30, and 240 s, respectively, plus an extension of 10 s/cycle after the first 10 cycles. An initial denaturation step of 94°C for 1 min and a final extension step of 68°C for 7 min were also included. The following primers were used for amplification of H-Sema-L and M-Sema-L: Est1, agccgcacacggtgcttttc; Est2, gcacagatgcgttcttgccc; Est3, accatagaccctggtgtccc; Est4, gcagtgatgctgccaccaac; Est6, acatgaggcaaccgtggcag; Est7, aggtagaccttgccacgtcc; Est13, ccattaatccagccgagccacacaag; Est14, catctacagctccgaacggtcagtg; hs5, gggatgacgcctcctccgcccgg; hs3, aagcttcacgtggaccagcaagccaagagtg; hs3c, aagctttttccgtccttccgtccgg. Adapter primers for RACE: MT-Ap1, ccatcctaatacgactcactatagggc; MT-Ap2, actcactatagggctcgagcggc; and cDNA synthesis primer, ttctagaattcagcggccgctttttttttttttttttttttttttttttvn. Primers for amplification of λ-gt10 inserts were 5gt10, agcaagttcagcctggttaagt; and 3gt10, ttatgagtatttcttccaggg.

Hybridizations and library screening. Lambda gt10 cDNA libraries from human spleen (Catalog No. HL5011a) and murine testis (Catalog No. ML1020a), primed with oligo(dT) and random oligonucleotides, were purchased from Clontech (Heidelberg, Germany). Plaques were screened according to the recommendations of the manufacturer using a radiolabeled 0.8-kb PCR fragment amplified with primers Est1 and Est3. Labeling with [α-^{32}P]dATP or -dCTP was performed by random priming (Feinberg and Vogelstein, 1983). The lambda inserts from purified plaques were amplified by long-distance PCR with primers 3gt10 and 5gt10 flanking the *Eco*RI cloning site, and their ends were sequenced. A multitissue Northern blot (Clontech) and a RNA dot blot containing various human RNAs (Clontech) were screened with the 0.8-kb probe. Hybridizations were carried out for 16 h under stringent conditions in 5× SSC, 50 mM sodium phosphate, pH 6.8, 50% formamide (FA), and 100 μg/ml total yeast RNA at 42°C, washed stringently at 65°C, with 0.2× SSC, 0.1% SDS. Filters were then exposed to Fuji BAS2000 PhosphorImager plates for 3 days.

Cloning procedures. The PCR-amplified fragments were gel-purified with a silica-based method (Qiaquick Kit, Qiagen, Hilden, Germany), ligated into the T-tailed plasmid pCR2.1 (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* K12 JM109 (Life Technologies, Gaithersburg, MD) by electroporation. Specific clones were identified by color selection with isopropyl-β-D-thiogalactopyranoside plus 5-bromo-4-chloro-indolyl-β-D-galactopyranoside and subsequent sequencing of their ends using standard primers. DNA was prepared by a standard alkaline lysis protocol (Ausubel *et al.*, 1994) or further purified by anion-exchange chromatography (Qiagen Midi Kit).

Nucleotide sequence determination. DNA sequencing was performed using an ABI 377 automated sequencer and *Taq* dyedeoxy terminator chemistry (Applied Biosystems, Foster City, CA). Double-stranded templates were sequenced using standard primers flanking the plasmid cloning sites and insert-specific oligonucleotides. All oligonucleotides were purchased from Eurogentec (Seraing, Belgium). The assembly of sequence readings was performed with the program XBAP (Dear and Staden, 1991) on a Sparcstation 10 (Sun Microsystems, Mountain View, CA). Oligonucleotides were selected using the program OSF (Hillier and Green, 1991) implemented in XBAP.

Nucleotide and protein sequence analysis. The GCG package (Genetics Computer Group, 1991) with the FASTA (Pearson and Lipman, 1988) and the BLAST programs (Gish and States, 1993; Altschul *et al.*, 1990) was used for analysis of nucleotide and amino acid sequences and for comparison with GenBank and SwissProt databases (versions 102.0 and 34.0, respectively). Putative signal sequences were analyzed with the SignalP server (http://www.cbs.dtu.dk/services/SignalP), using a neuronal network trained on eukaryotic sequences (Nielsen *et al.*, 1997). An initial progressive multiple alignment was performed with Clustal W (Thompson *et al.*, 1994) and further refined manually with the help of SEAVIEW (Galtier *et al.*, 1996). Evaluation of phylogenetic distances by neighborhood-joining analysis and bootstrapping was accomplished with Clustal W (Thompson *et al.*, 1994).

Fluorescence in situ hybridization (FISH). FISH was carried out according to standard protocols. Briefly, human and mouse metaphases were prepared from peripheral blood of a healthy male donor and from the mouse cell line BINE4.8 (Keyna *et al.*, 1995). The mouse cell line and the peripheral blood cells were allowed to incorporate 5-bromo-2-deoxyuridine (BrdU) for 16 h prior to preparation of the chromosomes. BrdU incorporation results in an enhanced DAPI banding pattern when the chromosomes are counterstained with this dye as a final step of the FISH procedure (Heng and Tsui, 1993). Slides were pretreated with RNase and pepsin to diminish disturbing plasma background as described (Liehr *et al.*, 1995). One hundred twenty or two hundred nanograms of the human or the murine nick-translated biotinylated semaphorin probes was hybridized per slide with human or mouse chromosomes, respectively, together with 4.0 μg of COT1-DNA and 20 μg of STD. A digoxigenin-labeled human chromosome 15-specific satellite DNA probe (Oncor, Gaithersburg, MD) was hybridized simultaneously to the human chromosomes. Hybridization was performed at 37°C for 3 nights in a humid chamber. After a postwashing series in 50% FA/2× SSC (three times 5 min at 45°C) and 2× SSC (three times 5 min at 37°C), the detection of biotinylated probe was performed with a FITC-avidin system (Liehr *et al.*, 1995). The chromosome 15-specific probe was detected with rhodamine-labeled anti-digoxigenin Fab fragments (Boehringer Mannheim). Slides were evaluated on a Zeiss axiophot fluorescence microscope. Images were captured with the ISIS digital FISH imaging system (MetaSystems, Altlussheim, Germany) using a XC77 CCD camera with on-chip integration (Sony). Twenty-five metaphases per probe were taken into account to determine the precise localization; each experiment was carried out twice.

RESULTS

Expression of the New Semaphorin Is Not Restricted to Neuronal Tissue

The presence of semaphorin-like molecules in both poxviruses (Kolodkin *et al.*, 1993) and a herpesvirus (Ensser and Fleckenstein, 1995) suggested that there may be distinct immunoregulatory functions for semaphorins, in addition to their described role in neuronal growth cone guidance. When we compared the herpesviral AHV-Sema protein sequence to recent updates of the human database of expressed sequence tags (dbEST), newly entered human ESTs surprisingly have a significantly higher homology to the viral semaphorins than to the previously described human sequences. We studied the expression pattern and complete cDNA and genomic structures of the respective human gene. Based on the EST sequences, oligonucleotide primers were designed for PCR and RACE. Two cDNA fragments of 0.8 and 0.6 kb were amplified with primer pairs Est1/Est3 and Est4/Est6, respectively.

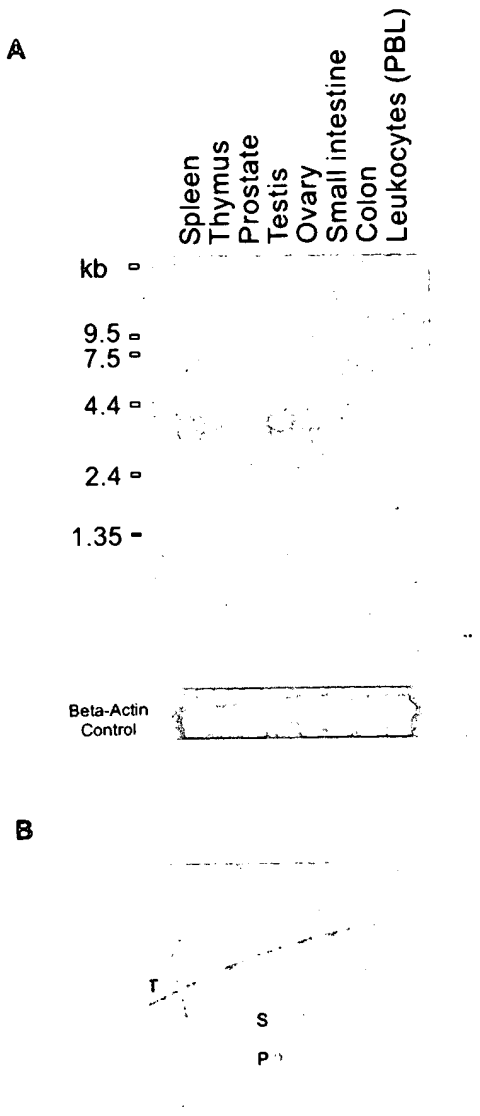

FIG. 1. Tissue-specific expression of H-Sema-L. (**A**) Multiple-tissue Northern blot containing 2 μg poly(A) RNA in each lane from spleen, thymus, prostate, testis, ovary, small intestine, colon mucosa, and peripheral blood leukocytes (from left to right) (Clontech, Heidelberg). Size markers are indicated. (**B**) RNA Master blot (Clontech, Heidelberg) containing RNA from various tissues. The amounts of RNA are normalized for the expression of several housekeeping genes. Significantly elevated signals are indicated. (T, testis; S, spleen; P, placenta; first two rows, various neuronal tissues). Both blots were hybridized to the 0.8-kb H-Sema-L-specific probe (indicated in Fig. 2) under stringent conditions.

Each pair was derived from different ESTs, indicating that the ESTs originated from the same gene. The 0.8-kb fragment was radioactively labeled and used to probe a multitissue Northern blot containing poly(A)-selected RNA (2 μg each) from various tissues (Figs. 1a and 2). The same probe was used to screen a dot-blotted panel of human RNAs normalized to the expression level of different housekeeping genes (Fig. 1b). Expression of a RNA of 3.2 kb was found predominantly in spleen, thymus, testis, and ovary on the poly(A) Northern blot. The signals were considerably weaker than the signals obtained with a labeled β-actin control probe (Fig. 1a). The expression pattern was confirmed on the dot blot, where elevated signals were detectable in placenta, spleen, and gonadal tissue, but not in neuronal or muscular tissue (Fig. 1b).

Molecular Cloning of H-Sema-L

After the tissue specificity of the H-Sema-L expression was determined, we decided to screen bacteriophage λ cDNA libraries from suitable tissues. Two independent screening experiments of about 3.5×10^5 λ clones yielded several hybridizing plaques that were purified to homogeneity by subsequent plaque purifications. PCR-amplified inserts were directly sequenced. Plaque λ5-1 contained the putative amino-terminus of the reading frame coding for the new semaphorin (Fig. 2).

RACE–PCR was performed simultaneously with the lambda screening to determine the 5′ and 3′ ends of the placental cDNA. Amplification products were cloned and sequenced. The 5′ RACE product was found to agree with the results of the lambda screening and sequencing, as it extended the 5′ sequence of λ5-1 by only a few nucleotides. This indicates that the actual 5′ end of the cDNA is represented. The sequence obtained by 3′ RACE is shown in Fig. 2. No other RACE products indicative for differential splicing were found. After the sequence of λ5-1 was supplemented by RACE, several incomplete λ clones were identified by hybridization. None of them contained differentially spliced 3′ cDNAs (data not shown). After the cDNA sequence was determined, the complete reading frame was PCR-amplified from placental cDNA in two segments (Fig. 2). Four independent clones from each segment were sequenced, and two clones that were free of PCR-introduced errors were joined to give the full-length cDNA sequence, which was again verified by sequencing.

Analysis of the H-Sema-L Coding Sequence and Amino Acid Sequence

The 2.6-kb cDNA codes for a polypeptide of 666 amino acids, starting at an ATG complying with Kozak's rules (Kozak, 1984) (Fig. 3). The nonglycosylated, unprocessed form has a calculated molecular mass of 74.8 kDa. Analysis with Signal P (Nielsen *et al.,* 1997) confirmed the presence of a putative signal peptide with a predicted cleavage site between residues 44 A and 45 Q. The unglycosylated protein has a MW of 70.3 kDa after signal peptide cleavage. The Sema domain starts after the cleavage site and continues to approximately residue 545. The positions of the cysteine residues within the Sema domain are nicely conserved compared to both viral and cellular semaphorins. An Ig-like motif [(Y,F)x1-2Cx(A,V)x(H)] is also well preserved among the semaphorins. The carboxy-terminal hydrophobic domain lacks a significant intracellular tail, thereby distinguishing this novel semaphorin from the other membrane-anchored semaphorins containing cy-

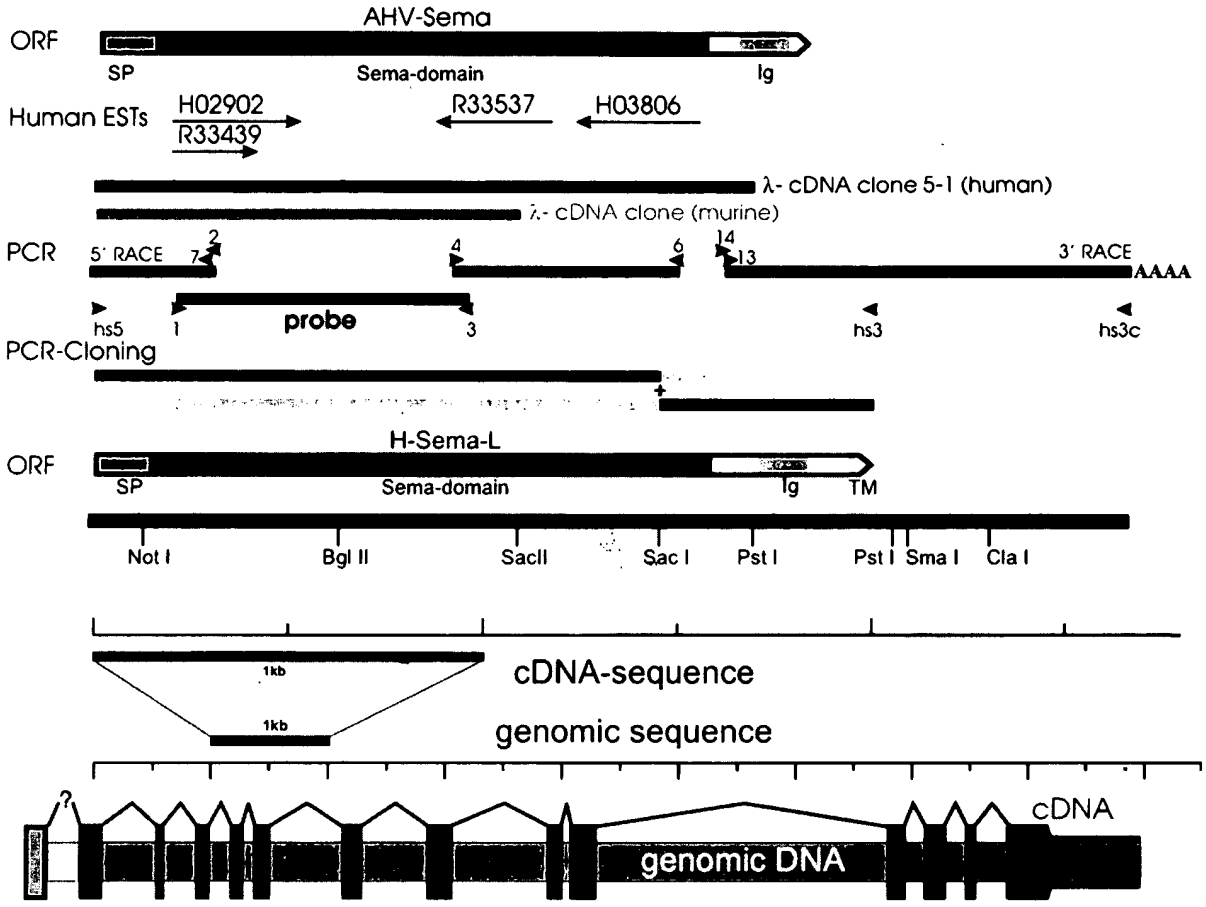

FIG. 2. Cloning of H-Sema-L cDNA and genomic organization. (**Top**) Localization of ESTs (designated by the respective GenBank accession numbers) relative to the AHV-Sema sequence is shown. The amplified PCR and RACE products as well as the position of the λ cDNA clones are shown. The PCR fragment used for hybridization (Fig. 1) is marked as probe. Positions of primers used for amplification are shown (➤, primer number; e.g., ➤ 1 denotes Est1). (**Middle**) Location of the H-Sema-L open reading frame on the cloned cDNA. (**Bottom**) The relative positions of the H-Sema-L exons on the genomic sequence are shown with appropriate scales.

toplasmatic tails (Hall *et al.*, 1996; Herold *et al.*, 1996). Analysis for common protein modification sites further revealed several N-linked glycosylation sites, myristoylation sites, and a RGD motif (Fig. 3).

Murine Homologue and Phylogenetic Analysis

Based on the newly discovered H-Sema-L, another search of dbEST revealed a small fragment of the murine homologue, which was termed M-Sema-L. Using the human 0.8-kb H-Sema-L probe for screening a λ gt10 cDNA library from murine testis, several hybridizing clones were isolated. Sequences were obtained as described above, and one clone contained the entire 5′ end of the M-Sema-L cDNA (Fig. 2). Two multiple sequence alignments were generated, one for all available semaphorins and another for the more closely related membrane-spanning H-Sema-L, the T-cell semaphorin CD100, the secreted neuronal H-Sema-III, and the viral semaphorins. The second methionine codon of AHV-Sema (position 48) was considered the relevant translational start site (Ensser and Fleckenstein, 1995), and the resulting shorter sequence was used for phylogenetic analysis. H- and M-Sema-L together with the viral semaphorins form a significantly distinct group from the other semaphorin members (Fig. 4).

Genomic Structure of H-Sema-L

Genomic structure and localization are known for few of the cellular semaphorins only. We determined the intron–exon structure of H-Sema-L from PCR-amplified genomic DNA. Fragments were cloned, and a total of 8.9 kb genomic sequence was determined. Comparison with the cDNA showed that the mRNA is a multiply spliced transcript consisting of at least 13 exons within the sequenced region (Fig. 2, bottom). PCR from genomic DNA with primers hs5 and Est2 or Est7 binding within the first 220 nt of the cDNA sequence was not able to amplify a corresponding genomic fragment, suggesting another large intron in the 5′ region of the gene.

Chromosomal Mapping of H-Sema-L and M-Sema-L

The semaphorin genes have been mapped on human chromosome 15q22.3–q23 and on murine chro-

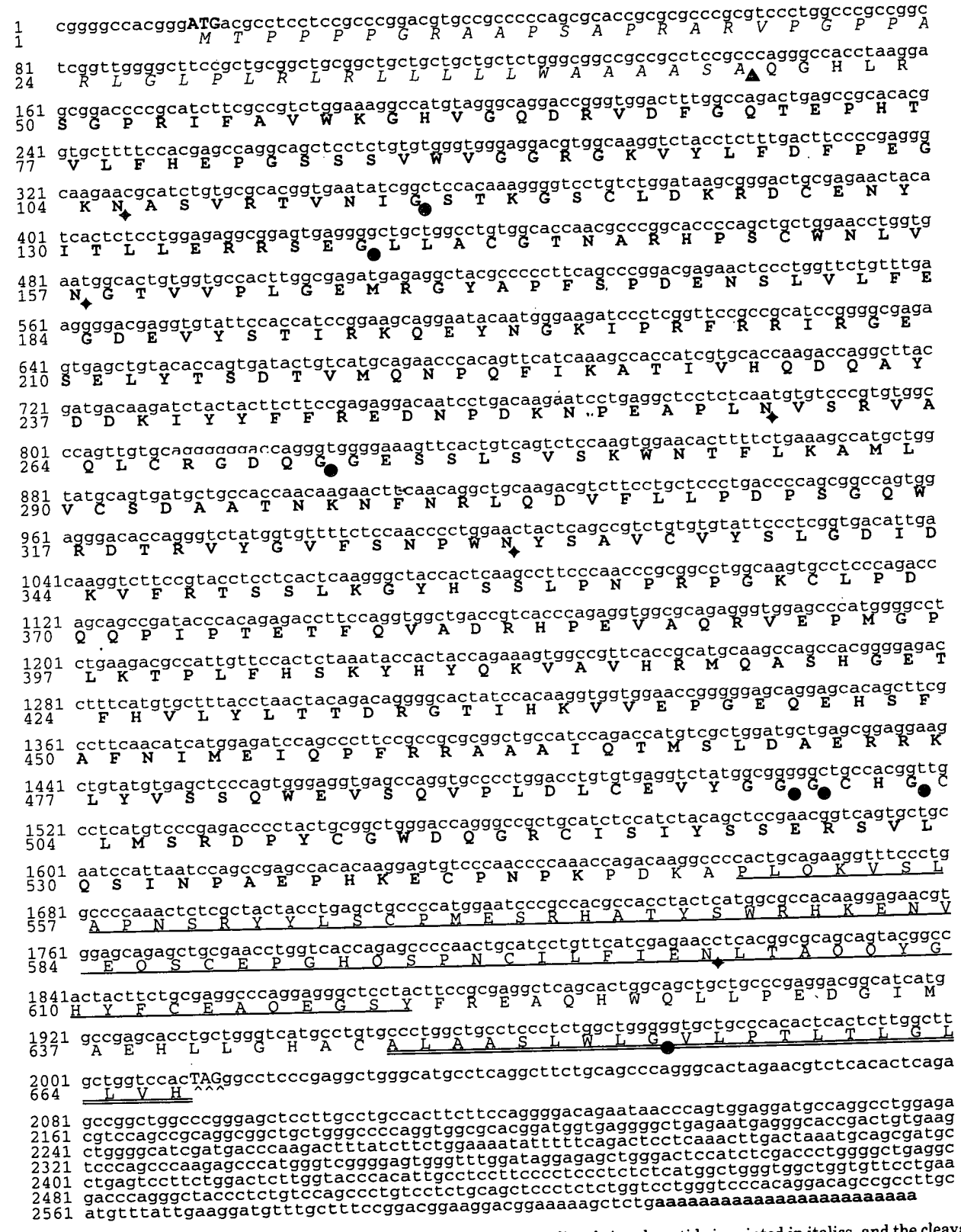

FIG. 3. H-Sema-L encoding cDNA sequence and protein sequence. The predicted signal peptide is printed in italics, and the cleavage site is indicated (▲). The Sema domain is shown in bold face type, the Ig-like motif is underlined, and the putative transmembrane region is double underlined. Putative N-linked glycosylation sites (◆), myristoylation sites (●) and the RGD motif (shaded box) are indicated.

mosome 9A3.3-B by means of FISH (Fig. 5). The mapping data obtained with the human and murine semaphorin genes are in good concordance with previously published comparative mapping studies of the respective genome regions (Imai and Kingsley, 1994).

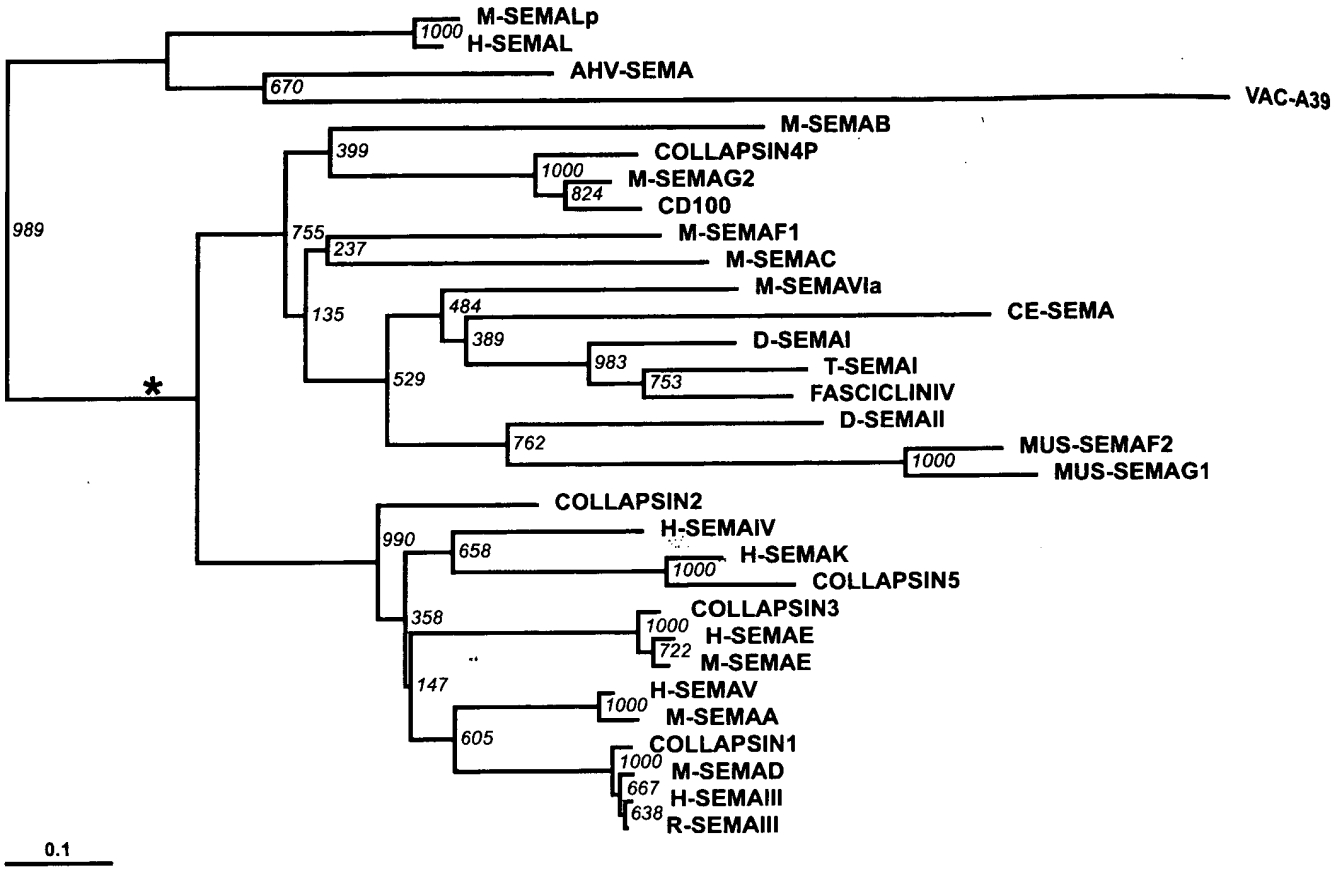

FIG. 4. Unrooted phylogenetic tree derived from multiple alignment of semaphorins present in the database (Table 1). An initial ClustalW alignment (Thompson *et al.*, 1994) was manually edited with SEAVIEW (Galtier *et al.*, 1996) and then reevaluated and bootstrapped (1000 reiterations) with ClustalW. Bootstrap values are shown at the appropriate branching sites. The outgroup used for displaying the tree is indicated by an asterisk.

DISCUSSION

Viral Semaphorins as Modulators of the Immune System

AHV-1 is a rhadinovirus (γ2-herpesvirus) that causes malignant catarrhal fever of ruminants. This usually fatal disease is associated with perivascular and epithelial lymphoid infiltrations in diseased animals (Reid and Buxton, 1989). The pathology of MCF is remarkably similar to the lymphoproliferative syndromes caused by herpesvirus saimiri, the prototype of γ_2-herpesviruses (rhadinoviruses) (Albrecht *et al.*, 1992). Upon sequencing the AHV-1 genome, we found an open reading frame with significant homologies to semaphorin genes and termed the gene AHV-Sema (Ensser and Fleckenstein, 1995; Ensser *et al.*, 1997). While truncated genes of similar structure have been found in poxviruses, this is the first known example of a semaphorin-like gene in a herpesvirus. Sequence alignment of the virus-encoded and vertebrate semaphorins revealed a number of conserved motifs and was consistent with the general design of semaphorins (Kolodkin *et al.*, 1993). Poxviruses and gammaherpesviruses code for numerous modulators of the immune and complement system (Ahuja and Murphy, 1993; Nicholas *et al.*, 1992; Smith *et al.*, 1990; Swaminathan *et al.*, 1993; Upton *et al.*, 1992; Albrecht and Fleckenstein, 1992; Isaacs *et al.*, 1992; Rother *et al.*, 1994; Yao *et al.*, 1995; Fodor *et al.*, 1995; Moore *et al.*, 1996; Neipel *et al.*, 1997; Arvanitakis *et al.*, 1997). Molecules from large DNA viruses that interfere with neuronal development have never been identified. Thus, it has been proposed that the viral semaphorins may play a role in the immune system as natural immunosuppressants that are secreted by virus-infected cells (Kolodkin *et al.*, 1993; Ensser and Fleckenstein, 1995).

Distinct Structure and Expression of the New Semaphorin

When ESTs with significantly higher homology to the viral semaphorins than to the known cellular semaphorins were discovered, we speculated that these might represent the presumptive targets of viral semaphorin action, either by mimicking or competing their functions. The complete human gene was cloned and characterized. In two parallel approaches by RACE–PCR from an adaptor-linked, double-stranded placental cDNA, and by screening a bacteriophage λ cDNA library from spleen, the complete cDNA of the new gene was cloned, which we termed H-Sema-L (Fig.

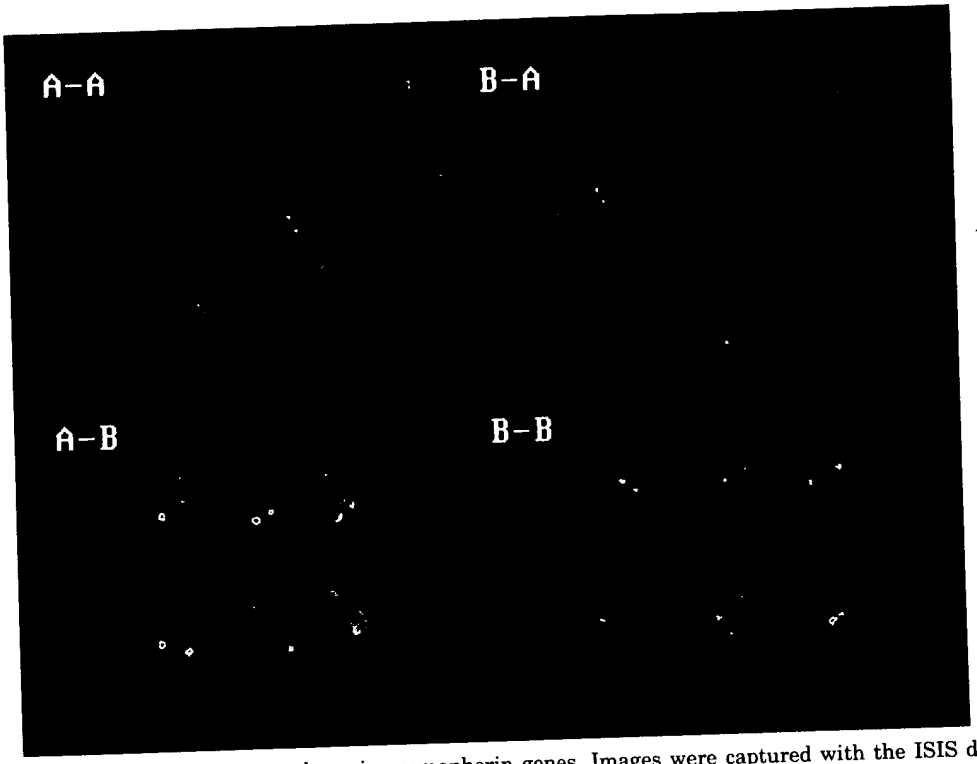

FIG. 5. Chromosomal mapping of human and murine semaphorin genes. Images were captured with the ISIS digital FISH imaging system (MetaSystems, Altlussheim, Germany) using a XC77 CCD camera with on-chip integration (Sony) on a Zeiss Axiophoto microscope. (**A-A**) A human metaphase and (**A-B**) six chromosomes 15 (from five different metaphases) hybridized with the human semaphorin probe and the chromosome 15-specific satellite DNA probe are shown; all chromosomes 15 exhibit semaphorin gene-specific signals in the subband q22.3–q23 and satellite DNA-specific signals in the centromere region. (**B-A**) A murine metaphase and in (**B-B**) six chromosomes 9 (from five different metaphases) hybridized with the murine semaphorin probe are shown; all chromosomes 9 exhibit signals in the subband A3.3-B.

2). As both methods produced similar results with respect to the 5′ end of the cDNA, it is likely that it represents the true transcriptional start region of the gene. The 2.6-kb cDNA contains an open reading frame that can code for a protein of 666 aa. The presence of a putative signal peptide (aa 1 to 44) in the translated protein, similarly present in all known semaphorins, is further support that the complete protein is encoded by the cloned cDNA. The typical semaphorin structure with an N-terminal signal peptide, a Sema domain, an Ig-like-domain, along with several N-linked glycosylation sites suggests that the mature peptide is a glycoprotein anchored to the cell surface by its hydrophobic transmembrane sequence (Figs. 2 and 3). The presence of the C-terminal hydrophobic transmembrane sequence without an intracellular tail in H-Sema-L is unique among the vertebrate semaphorins and defines a new class of semaphorins. The tissue specificity of the H-Sema-L expression was determined and revealed a unique expression pattern of the new gene differing from all known semaphorins. Expression was limited mainly to lymphoid tissues such as spleen and thymus, as well as gonadal tissue and placenta. Notable neuronal or muscular expression was not detected (Fig. 1). Although there are no indications for other transcripts in PCR or Northern blot, alternative splicing might lead to secreted forms or additional intracellular sequences.

Relationship to Known Semaphorins

The known semaphorins have been classified according to their carboxy-terminal structure (Püschel *et al.*, 1995; Culotti and Kolodkin, 1996). The observed homologies between semaphorins are in the range of 80–90% amino acid identity among closely related genes, i.e., homologues from different species within one subgroup like collapsin-1, H-Sema-III, and M-Sema-III. More distant relationships are found for different genes from the same subgroup in a single species, like M-Sema-A and -E or M-Sema-B and -C, which share amino acid identities of 43 and 37%, respectively. Genes from different semaphorin subgroups and different species generally have around 30% or less identical amino acids. The homology of the Sema domain alone, which is both the largest and most conserved part of these genes, is usually higher than the overall homology. Homology of H-Sema-L is highest to AHV-Sema, with 46% identity for the entire polypeptide and 53% identity within the Sema domain. The partial M-Sema-L amino acid sequence is 93% identical to that of H-Sema-L, clearly indicating that it represents the homologous murine gene. Homology to all other known semaphorins is in the range of 30% or less (data not shown). The phylogenetic tree resulting from analysis of a multiple alignment of all semaphorins clearly shows that most genes fall into distinct subgroups. This is determined mainly by the Sema

domain and the carboxy-terminal domain structure, as the secreted vertebrate semaphorins localize on one major branch (H-Sema-III-, IV, -V, -E, and their homologues) and the transmembrane semaphorins on another (e.g., CD100, M-Sema-B, and -C). The insect and nonvertebrate semaphorins, although of similar structure, are rather distantly related, and some murine semaphorins have a very unique structure, such as the thrombospondin motifs in M-Sema-F2 and M-Sema-G1. They most likely localize on different branches, but due to their low homology, phylogenetic analysis could not determine the exact branching order at a significant level.

H-Sema-L and its murine homologue M-Sema-L are located on a significantly distinct branch, together with the viral AHV-Sema. This may indicate an analogous function that is most likely distinct from that of the neuronal semaphorins. In fact, AHV-Sema is the next relative to the new genes. It is similar in size but does not have the transmembrane sequence. The viral protein might be secreted by virus-infected cells to attach to a receptor for H-Sema-L homologues and exert either an analogous or an antagonistic function.

Gene Structure and Genomic Localization

When sequencing the genomic DNA, we found that the H-Sema-L mRNA is a multiple spliced transcript with at least 14 exons. The last exon comprises the complete, unspliced 3′ region (Fig. 2). The genomic structure is known for only H-Sema-IV (GenBank Accession No. AC000063) and H-Sema-V (GenBank Accession No. U73167) located on chromosome 3p21.3. Both genes are also multiply spliced, with 18 and 17 exons in H-Sema-IV and H-Sema-V, respectively. Genomic localization by FISH resulted in a H-Sema-L-specific signal on chromosome 15q22.3–q23, close to the genomic loci of Tay-Sachs disease (15q23) and Bardet-Biedl syndrome (15q24). However, these diseases are not associated with known semaphorin gene functions and do not provide any hints as to putative H-Sema-L function. Interestingly the common breakpoint for the typical translocation t(15,17) in acute promyelocytic leukemia (APL) maps to 15q22. This translocation results in fusion of the amino-terminal "ring finger" motif and dimerization domain of the promyelocytic leukemia (PML) gene to the DNA-binding domain and retinoic acid-binding domain of the retinoic acid receptor-α (RARα) protein from chromosome 17q12 (Warrell *et al.,* 1993). On rare occasions fusions of RARα to the PLZF (Chen *et al.,* 1993), NPM (Redner *et al.,* 1996), or NuMA genes (Wells *et al.,* 1997) were found in APL. The murine homologue M-Sema-L was also mapped by FISH and found in the region homologous to 15q22.3–q23 in the murine genome, namely on mouse chromosome 9A3.3–B (Imai and Kingsley, 1994). Thus, localization of the genes is evolutionary preserved.

Most viral homologues to cellular genes have functions in the modification of apoptotic pathways or host immune response. As the known neuronal semaphorins function as repulsive signals, immunomodulation may be an alternative function for H-Sema-L. This is supported by the presence of an RGD motif similarly present in integrins and the membrane targeting sequence of the presumed H-Sema-L glycoprotein. Thus, in summary, we have cloned cDNAs of new human and murine homologues closely related to the viral semaphorin genes. The novel cellular semaphorins map to homologous loci in the human and murine genome, indicating an evolutionary preserved function. Their distinct protein structure and expression pattern suggest novel, probably immunomodulatory functions of these new eukaryotic genes and their viral homologues.

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Human Semaphorin K1 Is Glycosylphosphatidylinositol-linked and Defines a New Subfamily of Viral-related Semaphorins*

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The semaphorin family contains a large number of secreted and transmembrane proteins, some of which are known to act as repulsive axon guidance cues during development or to be involved in immune function. We report here on the identification of semaphorin K1 (sema K1), the first semaphorin known to be associated with cell surfaces via a glycosylphosphatidylinositol linkage. Sema K1 is highly homologous to a viral semaphorin and can interact with specific immune cells, suggesting that like its viral counterpart, sema K1 could play an important role in regulating immune function. Sema K1 does not bind to neuropilin-1 or neuropilin-2, the two receptors implicated in mediating the repulsive action of several secreted semaphorins, and thus it likely acts through a novel receptor. In contrast to most previously described semaphorins, sema K1 is only weakly expressed during development but is present at high levels in postnatal and adult tissues, particularly brain and spinal cord.

The semaphorins constitute a large family of evolutionally conserved glycoproteins that are defined by a characteristic semaphorin domain of approximately 500 amino acids (1–3). The first vertebrate semaphorin, collapsin-1 in chick, was identified by its ability to induce growth cone collapse (4). Consistent with this function, its mammalian homologue, sema III, has been shown to repel specific subsets of sensory axons (5). As a result of these and other studies, Coll-1/sema III/D has been implicated in the patterning of sensory axon projections into the ventral spinal cord and cranial nerve projections into the periphery (6–11).

Several other semaphorins have also been implicated as repulsive and/or attractive cues in axon guidance, axon fasciculation, and synapse formation (1, 12–17). In addition, members of the semaphorin family have been implicated in functions outside the nervous system, including bone skeleton and heart formation (9), immune function (18, 19), tumor suppression (20–22), and conferring drug resistance to cells (23).

Recent studies have identified the first semaphorin receptor as a member of the neuropilin family. Neuropilin-1 is a high affinity receptor for sema III, E, and IV, whereas neuropilin-2 binds differentially to the subfamily of secreted semaphorins (24–27).

The vertebrate semaphorin family can be classified into several phylogenetically distinct subfamilies (Ref. 15, Fig. 1*B*). Each subfamily has a unique structural arrangement of protein domains. The secreted members of the semaphorin family contain a characteristic semaphorin domain at the NH_2 terminus, followed by an immunoglobulin (Ig) domain and a stretch of basic amino acids in the carboxyl-terminal region. Between the NH_2-terminal semaphorin domain and the transmembrane spanning region, the transmembrane semaphorins contain several alternative structural motifs, including either an Ig domain, a stretch of thrombospondin repeats, or a sequence with no obvious domain homology. Interestingly, semaphorin-like sequences have been identified in the genomes of poxviruses (1) and alcelaphine herpesvirus-1 (28). These virally encoded semaphorins occupy unique branches of the semaphorin phylogenetic tree. Although it has been suggested that these viral semaphorins must have vertebrate homologues, to date none have been reported. Here we report the identification of a GPI[1]-linked human semaphorin, semaphorin K1, which is highly homologous to the semaphorin encoded by alcelaphine herpesvirus-1. The expression and binding properties of sema K1 suggest that it could play an important role in both adult nervous system and in modulating immune function.

EXPERIMENTAL PROCEDURES

Cloning of Sema K1—Four human ESTs, R33537, W47265, R33439, H03806, and one mouse EST, AA260340, were identified that show highest homology with the semaphorin gene in alcelaphine herpesvirus-1 (AHV sema). Oligonucleotides corresponding to the sequences of human ESTs were used to amplify by PCR a cDNA fragment from a human placenta cDNA library (CLONTECH). This PCR fragment corresponds to the central portion of sema K1. The 3′ end was cloned by rapid amplification of cDNA ends using human placenta Marathon-Ready cDNA from CLONTECH (29). The remaining 5′ end was cloned by PCR amplification from a CLONTECH human brain λgt11 cDNA library using an internal primer from sema K1 and an anchor primer corresponding to the λgt11 vector sequence. A specific PCR product corresponding to the 5′ end was identified by Southern blot using sema K1 oligonucleotides as probes. Repeated effort has been made, but failed to yield the remaining 5′ end of cDNA. The full-length cDNA of human sema K1 except the region corresponding to the signal peptide sequence was independently cloned from CLONTECH human placenta λgt10 library by high fidelity PCR amplification and its DNA sequence reconfirmed.

Expression Constructs—Three expression constructs were made that allow the expression of recombinant proteins tagged with either a Myc-His tag at the carboxyl terminus (pEX-mh), an alkaline phosphatase tag at the amino terminus and a Myc-His tag at the carboxyl terminus (pEX-AP), or an Fc domain of human immunoglobulin at the carboxyl terminus (pEX-Fc). Similar expression constructs have been

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF071542.

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; AHV, alcelaphine herpesvirus; AP, human placenta alkaline phosphatase; PI-PLC; phosphatidylinositol-specific phospholipase C; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; EST, expressed sequence tags.

made for collapsins and semaphorins, and the resulting fusion proteins were shown to be fully functional (7, 10, 23, 24, 30, 31). The multiple cloning site of pSecTagA (Invitrogen) was excised with *Pme*I and *Nhe*I and cloned into pcDNA3.1 (Invitrogen) to make Myc-His tagged vector pEX-mh. This expression vector contains a signal peptide sequence from immunoglobulin κ chain for protein secretion. The cDNA for human placental alkaline phosphatase was PCR-amplified from pSEAP (CLONTECH) and cloned into the *Sfi*I site of pEX-mh maintaining the original reading frame to make the AP-tagged vector pEX-AP. The Fc domain of human IgG1 and an enterokinase cleavage site were PCR-amplified from Signal-pIgplus (Novagen) and cloned into the *Apa*I to *Pme*I sites of pEX-mh maintaining the original reading frame to make the Fc-tagged vector pEX-Fc. Various cDNAs for full-length sema K1, extracellular domain of sema K1 (residues starting from Gly-612 to the carboxyl-terminal end were deleted), sema III, and neuropilin-1 were PCR-amplified from cDNA libraries and subcloned into these expression vectors. The neuropilin-2 expression construct was kindly provided by Dr. Marc Tessier-Lavigne (25).

Cell Surface Staining—COS-7 cells were transiently transfected with the full-length sema K1 in pEX-AP vector using LipofectAMINE (Life Technologies, Inc.). Two days after transfection, cells were washed and treated with or without PI-PLC (Boehringer Mannheim) at 250 milliunits/ml for 1 h at 37 °C. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature. After phosphate-buffered saline wash, cells were incubated with a rabbit anti-AP antibody (Accurate Antibodies) at a dilution of 1:500 for 1 h followed by a Cy3-anti-rabbit antibody at a dilution of 1:200. The fluorescent images of the transfected cells were photographed in a Zeiss microscope using a 40× lens.

Western Blotting—COS-7 cells were transiently transfected with the full-length sema K1 in pEX-AP vector with LipofectAMINE (Life Technologies, Inc.). Cells transfected with the full-length CD100 in pEX-AP served as a control. Two days after transfection, cells were incubated with or without 250 milliunits/ml of PI-PLC (Boehringer Mannheim) for 1 h at 37 °C. Supernatants and cell lysates were collected and run on a 4–20% SDS-PAGE gel and the AP-tagged sema K1 protein was detected with a horseradish peroxidase-conjugated anti-alkaline phosphatase antibody.

Protein Expression—Stable 293-EBNA cell lines secreting Myc-His-tagged, AP-tagged, or Fc-tagged sema K1 and sema III were derived from transfection of various expression plasmids followed by G418 selection. Conditioned media from stably transfected cell lines were collected and were confirmed for the expression and integrity of recombinant proteins by Western blot using anti-AP, anti-Fc, or anti-Myc antibodies. SDS-PAGE gel demonstrated that sema K1-Fc fusion protein migrates as a dimer linked by the disulfide bonds in the Fc region, whereas the sema K1-mh and AP-sema K1 are monomeric (not shown). Approximately equal amount of AP- or Fc-tagged sema III and sema K1 fusion proteins as judged by Western blot were used in the ligand binding experiments. The amount of active sema III used for the ligand binding experiment was further quantified by a growth cone collapse assay and estimated to be over 80 collapsing units/ml (4, 7).

Ligand Binding Experiments—COS-7 cells were transiently transfected with full-length neuropilin-1 or neuropilin-2 expression constructs with LipofectAMINE (Life Technologies, Inc.). The expression of neuropilin-1 or -2 was confirmed using a monoclonal antibody 9E10 against the Myc tag at the carboxyl-terminal ends of both receptors. After 2 days of transfection, the cells were then incubated with supernatants containing approximately equal amount of sema III-Fc or sema K1-Fc for 1 h. After post-fixing in 1% paraformaldehyde for 10 min, the cells were heat-inactivated at 65 °C for 1 h to destroy the endogenous alkaline phosphatase activity. Cells were then incubated with alkaline phosphatase-conjugated anti-Fc antibody at 1:500 dilution for 1 h and processed for chromogenic AP enzymatic reaction.

For the immune cell staining experiment, P388D1 or RBL-2H3 cells were fixed in 1% paraformaldehyde for 10 min. The suspension cells (A20 and Jurkat) were washed in phosphate-buffered saline once and fixed in 1% paraformaldehyde for 10 min and then cytospun onto glass slides. After blocking for 30 min, AP-sema K1- or AP-sema III-containing supernatants were added to each well and incubated for 1 h. The cells were then post-fixed in 100% methanol for 10 min, and the endogenous AP activity was heat-inactivated at 65 °C for 1 h. Cells were then processed for chromogenic AP enzymatic reactions. AP alone was used as a negative control. For experiments in which sema K1-mh or sema III-mh was used to compete with AP-sema K1 or AP-sema III binding, respectively, sema K1-mh or sema III-mh was incubated with different cell lines for 30 min at room temperature prior to AP-sema K1 or AP-sema III incubation.

Northern Blot Analysis and in Situ Hybridization—A 298-base pair DNA fragment corresponding to the sequence of mouse EST AA260340 was PCR-amplified from a mouse cDNA library. This DNA fragment is predicted to encode a mouse homologue of human sema K1 based on the fact that it shares over 95% amino acid identity with the corresponding region of human sema K1. It was used as a probe in the Northern blot analysis and the *in situ* hybridization experiments. Northern blot was either purchased from CLONTECH (Fig. 4*A*) or prepared as follows (Fig. 4*B*). Adult mouse tissues were homogenized in Ultraspec™ RNA reagent (Biotecx), and total RNA was extracted and precipitated. Poly(A)$^+$ RNA was further selected using FastTrack 2.0 mRNA isolation kit (Invitrogen). Two-microgram poly(A)+ RNA from each tissue was run in formaldehyde gels and blotted onto positively charged nylon membranes (Boehringer Mannheim). DNA probes were labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech) using random prime labeling kit of Life Technologies, Inc. Hybridization was performed in NorthernMax Buffer (Ambion) following the manufacturer's instructions. *In situ* hybridization procedure was performed on cryostat sections of E11, E15 mouse embryos, and on brain and spinal cord sections of P3 and 5-week-old mice (C57BL/6J) as described (32). Tissues were fixed in 4% paraformaldehyde for 4 h at 4 °C and embedded in OCT embedding compound. 20-μm sections were cut and were treated with 1.0 μg/ml proteinase K for 15 min at 37 °C, 0.2 M HCl for 20 min, and then acetylated for 10 min with 0.1 M triethanolamine and 0.25% acetic anhydride. Sections were prehybridized for 1 h at 65 °C, then hybridized with digoxigenin-labeled probes (2 μg/ml) overnight at 55 °C. The hybridization buffer consists of 50% formamide, 5 × SSC, 10% dextran sulfate, 1 × Denhardt's, 0.25 mg/ml tRNA, 0.1 mg/ml single-stranded DNA. After hybridization, slides were washed with 0.2 × SSC for 60 min at 65 °C and detected with an AP-conjugated anti-digoxigenin antibody at a dilution of 1:2000.

RESULTS

Semaphorin K1 Is Highly Homologous to a Viral Semaphorin—In an effort to identify veterbrate homologues of viral semaphorins, we searched existing EST data bases against semaphorin-like sequences found in vaccinia virus and in alcelaphine herpesvirus-1 (AHV sema) using the BLAST algorithm (33). Four human and one mouse ESTs were identified, which encode novel sequences that were most homologous to AHV sema (28). PCR primers were designed based on the EST sequences and were used to obtain a 2.5-kilobase pair cDNA that encodes a candidate semaphorin gene. The cDNA contains all the human EST sequences and predicts a protein of 634 amino acids with a molecular mass of 71.5 kDa (Fig. 1*A*). This protein is named semaphorin K1 (sema K1), since sema A to J has been designated for other known semaphorins in mouse genome data base and sequence analysis indicates that sema K1 is the first member of a new semaphorin subfamily (see below). Hydropathy analysis of the predicted sema K1 sequence (34) indicates that the sema K1 sequence is slightly incomplete, lacking approximately half of the signal peptide sequence required for protein secretion (35). Consistent with this predication, the alignment between AHV sema and sema K1 also showed an eight-amino acid difference at the amino-terminal end of sema K1 (Fig. 1*A*). The hydropathy analysis of sema K1 also identified a long stretch of hydrophobic residues at the carboxyl-terminal end, which resembles a signal peptide sequence required for GPI anchorage (36). Thus, sema K1 is predicted to be the first GPI-linked membrane protein in the semaphorin family.

The sequence of sema K1 is closely related to that of AHV sema. Whereas the sema domain of sema K1 shares 50% amino acid identity with that of AHV sema, it shares less than 30% identity with that of other known semaphorins. In addition, 17 out of 18 cysteine residues and 4 out of 5 potential *N*-linked glycosylation sites are conserved (Fig. 1*A*). The homology extends throughout the entire amino acid sequences of sema K1 and AHV sema except at the carboxyl-terminal end, where only sema K1 contains the signal peptide sequence for GPI anchorage. Thus, sema K1 is predicted to be a GPI-anchored mem-

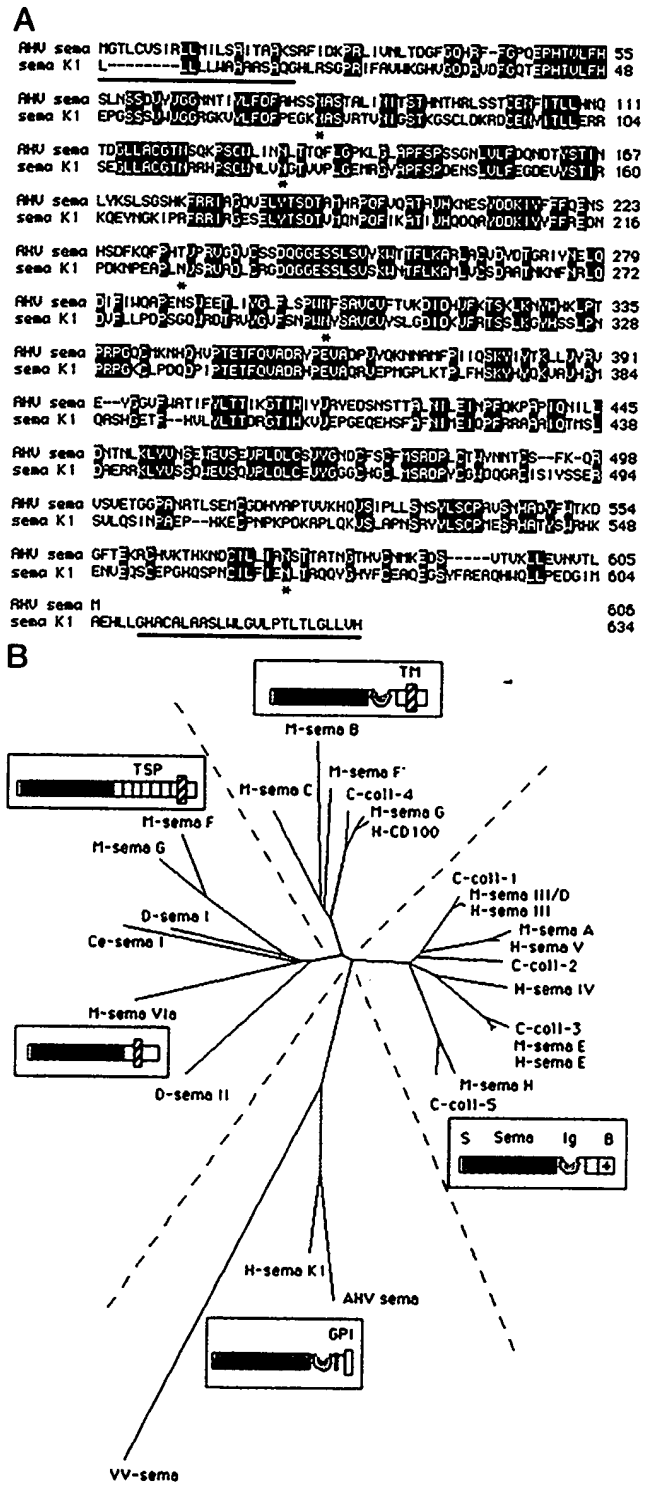

FIG. 1. **Structure of human semaphorin K1.** *A*, alignment of the amino acid sequences of sema K1 and viral AHV sema. The predicted NH_2-terminal and COOH-terminal signal peptide sequences are *underlined*. All asparagine residues likely to be glycosylated are marked by *asterisks*. *B*, dendrogram tree and the predicted structural arrangement of veterbrate semaphorins within each tree branch. A dendrogram tree has been constructed for the indicated semaphorins based on a sequence comparison of sema domains by Clustal W (49). The *dashed lines* separate the four major branches of the tree. Schematic diagrams displaying the domain structures of each subfamily of vertebrate semaphorins is shown within the boxes. *C*, chicken; *H*, human; *M*, mouse; *D*, *Drosophila*; *CE*, *Caenorhabditis elegans*; *AHV*, alcelaphine herpesvirus-1; *VV*, vaccinia virus; *S*, signal peptide; *Sema*, semaphorin domain; *Ig*, immunoglobulin-like domain; *B*, basic region; *TM*, transmembrane domain; *TSP*, thrombospondin repeats; *GPI*, GPI linkage.

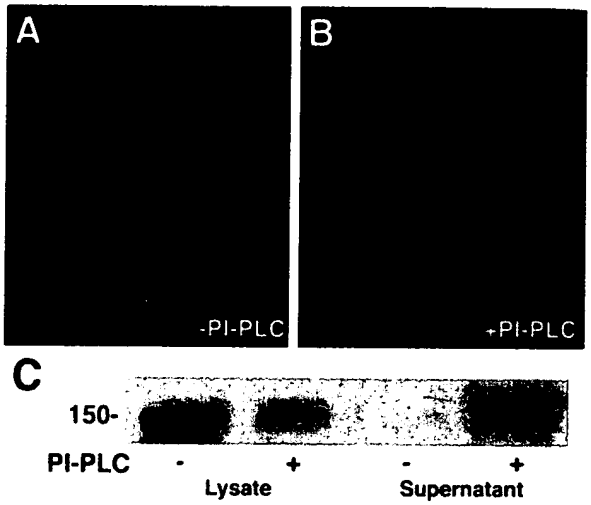

FIG. 2. **Effect of PI-PLC treatment on the cell surface expression of semaphorin K1.** COS-7 cells transfected with the full-length AP-sema K1 fusion construct were treated without (*A*) or with (*B*) PI-PLC. An anti-AP antibody and a Cy3-conjugated secondary antibody was used to detect the AP-sema K1 fusion protein on the cell surface. The cell supernatants and lysates of transfected COS-7 cells treated with or without PI-PLC were run on a SDS-PAGE gel (*C*). The fusion protein was detected with a horseradish peroxidase-conjugated anti-alkaline phosphatase antibody in Western blot. The molecular mass in kilodaltons is marked to the *left*.

brane protein, whereas AHV sema is likely to be a secreted protein. The unique structural arrangement of sema K1 suggests that it may define a new subfamily of vertebrate semaphorins. Consistent with this hypothesis, protein sequence homology analysis showed that sema K1 and AHV sema belong to the same branch of the dendrogram tree and this branch is distinct from that of other semaphorins (Fig. 1*B*). Sequence alignment with other semaphorins also revealed that members of the viral-related semaphorin subfamily lack three tryptophan residues conserved in other semaphorins, suggesting that viral-related semaphorins may contain a structurally distinct sema domain.

Semaphorin K1 Is a GPI-anchored Membrane Protein—To confirm that sema K1 is a GPI-anchored membrane protein, we have transfected COS-7 cells with a sema K1 expression construct and determined the localization of the expressed sema K1 protein. To track sema K1 protein expression, an AP-tagged version of sema K1 was engineered in which the human placenta alkaline phosphatase was fused to the full-length sema K1 at the NH_2 terminus. This fusion protein can be detected with an anti-AP antibody or AP activity. Upon transfection of the expression construct into COS-7 cells, the sema K1 fusion protein was detected on the surface of those transfected cells (Fig. 2*A*). Treatment with PI-PLC resulted in a complete removal of the fusion protein from cell surfaces (Fig. 2*B*). To examine whether the release of sema K1 fusion protein from cell surfaces is a specific action of PI-PLC rather than the result of random proteolysis, we compared the presence of this fusion protein in the supernatant and lysate of transfected COS-7 cells with or without PI-PLC treatment. Supernatants and lysates from PI-PLC treated or untreated cells were subjected to Western blot analysis. A 150-kDa protein corresponding to the predicted size of the fusion protein was detected with the anti-AP antibody. When the transfected COS-7 cells were not treated with PI-PLC, most, if not all, of the fusion protein was found to be associated with the cell lysate (Fig. 2*C*). Treatment of these cells with PI-PLC resulted in significant release of the fusion protein from the cell lysate into the supernatant, without apparent proteolysis (Fig. 2*C*). In a control experiment,

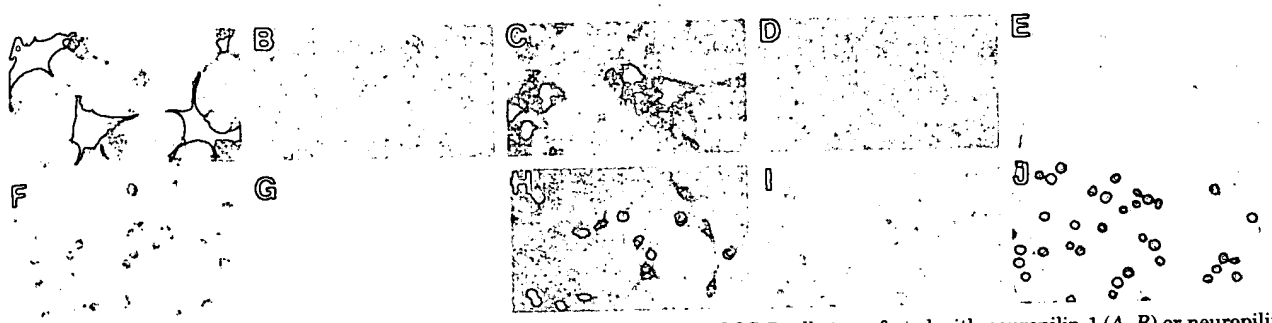

FIG. 3. **Binding of semaphorin K1 to neuropilins and immune cell lines.** COS-7 cells transfected with neuropilin-1 (*A*, *B*) or neuropilin-2 (*C*, *D*) expression constructs were tested for binding with sema III-Fc (*A*, *C*), or sema K1-Fc (*B*, *D*). P388D1 (*E–H*) macrophage and Jurkat T cells (*I*, *J*) were tested for binding with AP control (*E*), AP-sema K1 (*F*, *I*), AP-sema III (*H*, *J*). Binding of AP-sema K1 after preincubation with sema K1-mh is also shown (*G*).

PI-PLC treatment did not release the transmembrane semaphorin CD100 into the cell supernatant (not shown). Furthermore, when a stop codon was introduced immediately NH_2-terminal to the predicted signal peptide sequence for GPI linkage (residues starting from Gly-612 to the carboxyl-terminal end were deleted), the resultant sema K1 protein was released to the cell supernatant in the absence of PI-PLC (not shown). Thus, we conclude that sema K1 is attached to the cell membrane via a GPI linkage.

Semaphorin K1 Binds to Specific Immune Cell Lines—Neuropilin-1 and neuropilin-2 have recently been identified as receptors or components of a receptor complex for sema III and other secreted semaphorins (24–26). To determine whether sema K1 could use neuropilin-1 or -2 as its receptor, we tested the ability of sema K1 to bind COS-7 cells transfected with neuropilin expression constructs. Soluble sema K1 fusion proteins containing either an AP tag at the NH_2 terminus (AP-sema K1), an Fc domain of human IgG1 at the COOH terminus (sema K1-Fc), or a Myc-His tag at the COOH terminus (sema K1-mh) were produced and were used in the ligand binding assay. Similarly arranged AP-sema III, sema III-Fc, and sema III-mh fusion proteins were prepared as controls. To test for interactions with neuropilin-1 or -2, sema K1-Fc, or AP-sema K1 were incubated with neuropilin-expressing COS-7 cells, and ligand binding was detected using an anti-Fc antibody or a chromogenic AP enzymatic reaction. Under conditions where sema III-Fc binds to COS-7 cells expressing neuropilin-1 or -2, the dimerized sema K1-Fc does not bind to either (Fig. 3, *A–D*, note that sema III binds to neuropilin-2 with lower affinity than to neuropilin-1). In this experiment, sema III-Fc does not bind to mock-transfected COS-7 cells and Fc-tagged MUC18, an adhesion molecule served as a control, does not bind to the neuropilin-expressing COS-7 cells (not shown). Both served as negative controls. Similarly, under conditions when AP-sema III can bind to COS-7 cells expressing neuropilin-1 or -2, the monomeric AP-sema K1 does not bind to these cells (data not shown). Thus, sema K1 does not bind neuropilin-1 or -2 with high affinity and may not act through these receptors.

To determine whether or not the soluble sema K1 fusion proteins are competent to bind a cognate receptor and to provide an entry point for investigating the role of sema K1 in modulating immune function, we analyzed several immune cell lines for the presence of sema K1 binding sites. AP-sema K1 or AP-sema III were incubated with Jurkat T cells, A20 B cells, P388D1 macrophages, and RBL-2H3 mast cell lines and the bound ligands were detected with chromogenic AP enzymatic reaction (Fig. 3, *E–J*). AP-sema K1 bound only to the cell surfaces of P388D1 macrophage and RBL-2H3 mast cell lines. This binding is specific, since AP alone does not bind to any of the cell lines, and the binding could be competed by preincubation with sema K1-mh. In comparison, AP-sema III binding was detected on cell surfaces of all four immune cell lines tested. This binding is also specific, since preincubation of these cells with sema III-mh blocks the binding (not shown). The ability of sema III-Fc or sema K1-Fc to bind these four cell lines was also tested and similar results obtained (not shown). We conclude that sema III can bind the four immune cell lines tested, which contrasts with the more selective binding of sema K1 to macrophage and mast cell lines, suggesting the existence of a specific receptor for sema K1 in these cell lines.

Semaphorin K1 Is Preferentially Expressed in Postnatal and Adult Brain and Spinal Cord—To help define the biological role of sema K1, we examined the expression of sema K1 by Northern blot analysis and *in situ* hybridization. A 298-base pair cDNA corresponding to the mouse homologue of human sema K1 was used as a probe in these studies. This probe does not cross-hybridize with the mRNA of other semaphorins. Northern blot analysis of mRNA isolated from adult mouse tissues revealed a single sema K1 transcript at 4.4 kilobase pairs (Fig. 4). The sema K1 transcript is highly expressed in brain, spinal cord, lung, and testis; moderately expressed in heart, muscle, adrenal gland, lymph nodes, thymus, and intestine; weakly expressed in spleen and kidney; and not detectable in liver, bone marrow, and stomach.

To examine the distribution of sema K1 mRNA in detail, *in situ* hybridization analysis was performed on tissue sections of embryonic day 11 and day 15 embryos, and on the brain and spinal cord sections of postnatal day 3- and 5- week-old mice. A digoxigenin-labeled antisense RNA probe for sema K1 was used in this study. The sema K1 sense probe served as a control, which gave no significant hybridization signal on tissue sections of P3 and adult mice, but gave weak and uniform background signals in E11 and E15 tissue sections (not shown). Sema K1 mRNA does not appear to be expressed significantly in the developing mouse embryo, since no strong hybridization signals were detected in tissue sections generated from entire E11 and E15 embryos except within the spinal cord regions. Above background hybridization signal was detected in the ventral and lateral regions of the spinal cord at E11 (Fig. 5*A*) and E15 (Fig. 5*B*). At P3, the signal became more intense and expanded both dorsally and medially (Fig. 5*C*). By 5 weeks, strong hybridization signals were present in cells scattered throughout the gray matter except in the dorsal region where Rexed lamina layers I and II reside (Fig. 5*D*).

No significant expression of sema K1 mRNA is detected at E11 and E15 in the primodial cerebral cortex and cerebellum (data not shown). At P3, intense expression of sema K1 mRNA becomes evident in the marginal zone of the cerebral neocortex. Moderate levels of expression were detected in the cortical plate and subplate (Fig. 5*E*). In the brain of 5-week-old mice, the expression of sema K1 mRNA becomes widespread throughout the entire cerebral cortex. The level of mRNA ex-

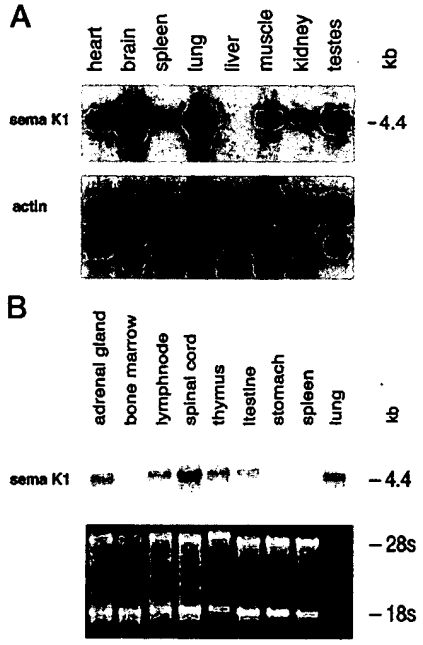

FIG. 4. **Northern analysis of sema K1 expression.** Northern blots made by CLONTECH (*A*) or from various adult mouse tissues (*B*) were probed with the 298-base pair mouse sema K1 probe. Actin or RNA staining serve as controls for the amount of mRNA loaded into each lane.

pression is moderate among all lamina layers except layer I, where no expression is evident (Fig. 5*F*). In the cerebellum at P3, sema K1 message is strongly expressed in the external germinal layer and the primodial Purkinje cell layer (Fig. 5*G*). By 5 weeks, intense expression of sema K1 mRNA is found only in the Purkinje cells (Fig. 5*H*). In addition to the dynamic patterns of expression in spinal cord, cerebellum, and cortex, sema K1 mRNA is found to be present in other structures of adult brain, including the cochlear nucleus, inferior colliculus, hippocampus and dentate gyrus, the olfactory glomerular cell layer and mitral cell layer, and thalamic structures (Table I).

DISCUSSION

GPI-linked Semaphorin K1 Defines a New Subfamily of Viral-related Semaphorins—We have identified a novel human semaphorin, sema K1, that is related to a semaphorin-like sequence in alcelaphine herpesvirus-1 (28). Sema K1 shares 45% amino acid identity with AHV sema but less than 30% with other known semaphorins. Moreover, sema K1 and AHV sema fall into the same branch of a dendrogram tree. Whereas our studies reveal a mammalian counterpart for viral AHV sema, equivalent mammalian homologues for poxvirus semaphorins are still unknown. Nevertheless, since the poxvirus semaphorins occupy a distinct branch of the phylogenetic tree, we suspect that poxvirus-related veterbrate semaphorins might also exist.

Many viruses encode proteins that can function as immune modulators, for example by interfering with antigen presentation, acting as cytokines or cytokine antagonists, inhibiting apoptosis, or interrupting the complement cascade (37). In the case of AHV, the semaphorin-like gene is found within a region of the viral genome that contains genes not conserved among the herpesviruses (38). In many viruses, nonconserved genes often encode homologues of cellular genes that are known to be involved in immunomodulation. For example, poxviruses encode a soluble form of tumor necrosis factor receptor (TNFR), which can bind to TNFα and TNFβ with high affinity (39). Mutation of this viral TNFR results in significantly attenuated virulence, suggesting that the viral TNFR may function to block the antiviral effects of TNF (40).

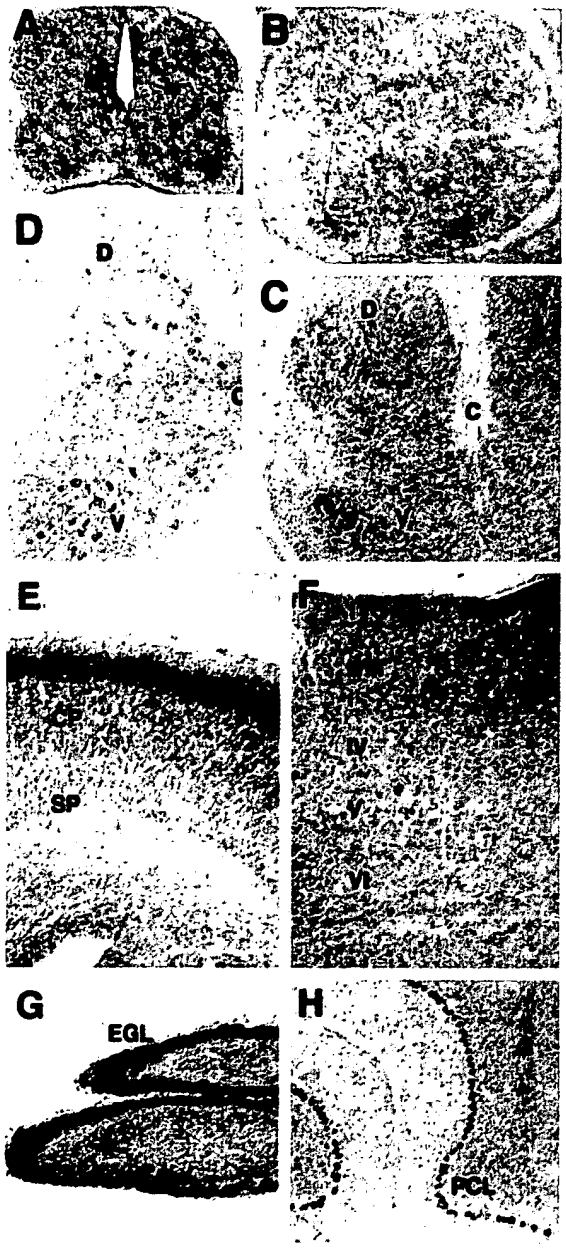

FIG. 5. **Sema K1 mRNA expression in spinal cord, cerebral cortex, and cerebellum.** Coronal sections of mouse spinal cord from E11 (*A*), E15 (*B*), P3 (*C*), and 5-week (*D*)-old mice, horizontal sections of cortex at P3 (*E*) and 5-week (*F*)-old, and cerebellum at P3 (*G*) and 5-week (*H*)-old mice were hybridized with digoxigenin-labeled antisense sema K1 probe. *D*, dorsal spinal cord; *V*, ventral spinal cord; *C*, central canal; *MZ*, marginal zone; *CP*, cortical plate; *SP*, subplate; *EGL*, external germinal layer; *PCL*, Purkinje cell layer. The *arrow* marks the primodial Purkinje cells.

Viral semaphorins have been proposed to be a part of a defense mechanism used by certain viruses to suppress the immune response of the host (1). Since sema K1 is highly homologous to AHV sema, it is tempting to speculate that vertebrate sema K1 could potentially play an important role in modulating immune function. Consistent with this hypothesis, sema K1 can bind to macrophage and mast cell lines. In addition, sema K1 mRNA is detected in several immune tissues, including lymph nodes, thymus, and spleen. A proposed role for semaphorins in immune function is not without precedent. CD100, a transmembrane semaphorin, can act as a T cell co-activation antigen and can promote B cell survival and aggregation (18, 19).

TABLE I
Expression of sema K1 mRNA in adult mouse brain

Moderate to high levels of sema K1 mRNA expression were detected in various regions of adult brain. The areas of sema K1 mRNA expression in the brain are listed.

Medulla	Brain stem (pons and cerebellum)	Midbrain	Diencephalon	Cerebral hemispheres	Olfactory bulb
Cochlear nucleus Gracile nucleus	Lateral lemiscus Spinal trigeminal nucleus Principal sensory trigeminal nucleus Medial lemniscus Purkinje cell layer Deep nuclei of cerebellum Pontine reticular formation	Inferior colliculus	Reticular nucleus Lateral geniculate	Hippocampus Dentate gyrus Cortex layers II-VI	Mitral cells Periglomerular cells

Sema K1 is the first known GPI-linked semaphorin. This conclusion is predicted by the presence of a carboxyl-terminal signal peptide required for GPI-anchorage and confirmed by the ability of PI-PLC to release membrane bound sema K1 into the supernatant. The structural arrangement of sema K1 contrasts with that of viral AHV sema which does not contain a carboxyl-terminal signal peptide sequence and is predicted to be a secreted protein. This structural difference between sema K1 and AHV sema may be of functional significance. It is possible that whereas GPI-linked sema K1 can provide an immune-modulating function in localized regions, the viral AHV sema may act in a diffusible fashion to subvert the host immune response in much broader areas. The identification of sema K1 as a GPI-linked protein demonstrates that members of the semaphorin family can exist in secreted, transmembrane, or GPI-linked forms.

Semaphorin K1 Binds to a Receptor Distinct from Neuropilin-1 or -2—The mechanism of action of sema K1 remains unknown. Neuropilin-1 is a receptor mediating the repulsive action of sema III (24, 25). Neuropilin-1 and -2 bind to members of secreted semaphorins (16, 25, 26). It was reported that sema III binds with high affinity to neuropilin-1 but not neuropilin-2, although it was pointed out that the possibility of sema III binding to neuropilin-2 with low affinity could not be excluded (26). Our study clearly demonstrates that sema III can indeed bind to neuropilin-2, albeit with apparent low affinity. Whether neuropilin-1 or -2 can bind to members of other subfamilies of semaphorins remains unknown. It has been reported that in initial experiments, sema VIa and *Drosophila* sema II do not interact with either of the neuropilins (26). We showed here that sema K1 does not interact with these neuropilins either. One explanation is that our recombinant sema K1 is not functionally active, but this appears unlikely since sema K1 can bind to macrophage and mast cell lines (but not T or B cell lines). This binding profile is different from that of sema III, which interacts with all the cell lines tested. These results support the notion that sema K1 possesses binding sites in macrophage and mast cell lines that are distinct from those of sema III, suggesting that sema K1 acts through an as yet unidentified receptor.

In this study, we showed that sema III possesses binding sites on T cell, B cell, macrophage, and mast cell lines. These results raise the possibility that sema III might have a role in immune modulation. One possibility is that sema III might function in the immune system in a manner similar to that in the nervous system, acting as a guidance cue to regulate lymphocyte migration and homing.

Functional Implications of Semaphorin K1 Expression—The abundant expression of sema K1 in brain and spinal cord suggests that sema K1 is likely to play an important role in the nervous system. Sema K1 is weakly expressed in developing embryos but abundantly expressed in postnatal and adult brain and spinal cord. The expression pattern of sema K1 stands in contrast with that of previously known semaphorins and neuropilins, which are strongly expressed in the developing nervous system (2, 8, 10, 15, 26, 41–46). Sema K1 expression is particularly striking in spinal cord, cerebellum, and cortex, where the expression pattern is dynamically regulated. In spinal cord, sema K1 expression contrasts with the expression pattern of sema III and sema VIa, which are expressed in similar regions of the ventral spinal cord embryonically, with expression significantly reduced by birth (3, 43, 45). It is possible that sema K1 might be active at later developmental stages when final innervation patterns are being formed. The expression pattern of sema K1 mRNA also contrasts with that of sema VIa in the cerebellum and cortex, where sema VIa is highly expressed in early cortical and cerebellar development but is greatly reduced by birth (45). The high level of sema K1 expression in the cerebellar external germinal layer and primordial Purkinje cells at P3, the stage of active granule neuronal migration, raises the possibility that sema K1 could regulate migration processes in the primordial cerebellum. Similarly, the presence of sema K1 mRNA in regions of marginal zone, cortical plate, and subplate at a time of active neuronal migration in the cortex is also potentially consistent with this proposed role.

The widespread and abundant expression of sema K1 mRNA in adult brain suggests that it could play a role in the maintenance and plasticity of connections in the adult nervous system. Many members of the semaphorin family are implicated as repulsive guidance cues that repel growing axons during development. If sema K1 is functionally analogous to those semaphorins, it could help maintain established neuronal circuits in the adult nervous system by inhibiting unwanted neurite sprouting. Regulated expression of sema K1 and its receptor in regions of plasticity could contribute to the remodeling of neuronal circuits. The presence of such maintenance factors in the adult brain may also contribute to the failure of central nervous system axons to regenerate upon injury. At present we do not know whether sema K1 can inhibit the extension of axons of adult central nervous system neurons.

The evidence that sema K1 binds to macrophage and mast cell lines and that high level of sema K1 mRNA expression is found in the adult brain also raises the possibility that sema K1 might be involved in neural-immune interaction. Of particular relevance is the possibility that sema K1 affects microglial cells, the resident macrophages in the brain. Microglial cells are thought to arise from the blood monocytes of the monocyte-macrophage series, which invade the brain during embryonic and postnatal life. Between the second and third postnatal week they transform into ramified resting microglia, which are marked by their diminished phagocytic activity and mitotic capability (47). The fact that sema K1 shows strong homology to a possible viral immune suppresser (AHV sema), is highly expressed in postnatal and adult brain when microglia become ramified, and contains binding sites in macrophage cell lines are all consistent with the hypothesis that sema K1 might contribute to the ramification of microglia and maintenance of

their resting state. Last, it has been known that nerve injury in the peripheral nervous system is always followed by rapid and massive invasion of macrophages, whereas macrophage infiltration to the injured areas of the central nervous system is very limited. The lack of macrophage infiltration may in part explain the failure of axon regeneration in the central nervous system (48). It is possible that members of the semaphorin family, including sema K1, could contribute to the limited migration and infiltration of macrophages into immune privileged sites such as brain. Thus, the possible regulation of lymphocyte migration and response both within and outside of the nervous system appear to represent reasonable biological targets for future investigation of sema K1 and sema III activity.

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